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Innate immunity and resistance to an emerging infectious disease in a wild bird

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Abstract

Innate immunity is expected to play a primary role in conferring resistance to novel infectious diseases. Despite this, few studies have attempted to examine its role in the evolution of resistance to emerging pathogens, instead concentrating on the role of acquired immunity (e.g. *Mhc* genes). Here we used experimental infections and cDNA microarrays to determine whether changes in the innate and/or acquired immune responses accompanied the emergence of resistance in eastern U.S. house finches (*Carpodacus mexicanus*) to a recent outbreak of conjunctivitis-causing bacterium (*Mycoplasma gallisepticum*- MG). Three days following experimental infection with MG, we observed differences in the transcriptional responses in spleens between House Finches from eastern or western US populations. In particular, birds from the western US, with no prior exposure to MG, down-regulated gene expression relative to controls, while those from the east, with a 12-year history of MG exposure, showed no expression change. This result is significant because, in poultry, MG is known to manipulate host immunity, suggesting that such manipulation also occurred in western birds only. Infected eastern birds then up-regulated genes associated with acquired immunity (cell-mediated immunity) 14 days after infection relative to controls, whereas birds from the western population retained similar expression patterns on day 14 as they did on day three. These observations indicate marked population differences in the temporal course of response to infection with MG, and suggest that innate immune processes were targets of selection in response to MG in the eastern U.S. population.

Introduction

Novel pathogens are powerful selective agents in humans (Diamond 1997) and other animals (Grenfell & Dobson 1995; Haldane 1949), and can have devastating effects on biodiversity (Benning *et al.* 2002; Lips *et al.* 2006). Studies simultaneously monitoring the emergence of an infectious disease in the wild and the associated changes in host populations are rare, leading to a reduced understanding of how hosts evolve immunity to novel pathogens, particularly in vertebrates. One exception involves the study of rapid evolution of disease resistance in European rabbit (*Oryctolagus cuniculus*) affected with myxomatosis in Australia (Kerr & Best 1998). The Myxoma virus was released in 1950 and spread rapidly throughout the susceptible Australian rabbit population. Within a few years however, resistance emerged, apparently mediated through escape from pathogen-induced immunosuppression which facilitated the development of an enhanced innate and then a specific cell-mediated immune response (Best & Kerr 2000). Although we know that wild vertebrate host populations can evolve resistance to novel pathogens rapidly (Bonneaud *et al.* 2011; Marshall & Fenner 1958), whether or not such resistance is mediated through initial changes to innate immunity as the study of rabbits would suggest is unclear.

Despite the potential for innate immunity to play a key role in the response to novel pathogens, the vast majority of studies in ecological immunology in vertebrates have focused on the acquired immune system (Acevedo-Whitehouse & Cunningham 2006; van der Most *et al.* 2011). The most likely reason for this trend is that most host-pathogen systems studied are assumed to be co-evolving. Unlike innate immunity, responses of acquired immunity are usually pathogen-specific and therefore represent a more targeted and effective defensive response, particularly against known pathogens (Janeway 2005). For example, of particular

interest in host-parasite co-evolution has been the role of the polymorphic *Mhc* genes in detecting foreign antigens and triggering pathogen-specific T-lymphocyte cytotoxicity and humoral immune responses (Piertney & Oliver 2006; Sommer 2005; Spurgin & Richardson 2010). However, during the early stages of infection, pathogen-specific recognition alleles may either be absent or at such low frequencies in host populations that such populations are ill-equipped to deal with novel pathogens. Under such conditions, the spread of adaptive alleles may thus be slow and stochastic (Hedrick 2002; Wright 1955). By contrast, innate immunity comprises immediate, non-specific immune processes that are triggered when pattern recognition receptors detect a limited repertoire of conserved but common microbial patterns (e.g., LPS) (Janeway 1989). As a result, innate immunity provides the first line of protection against most pathogenic attacks and can stem infections while pathogen-specific processes are being activated (Janeway 2005). As such, we might expect innate immunity to play a particularly important role during outbreaks of novel infectious diseases. This is particularly true of pathogens that are able to manipulate and avoid immune detection, since detection by the acquired immune system (e.g., by *Mhc* molecules) requires their prior recognition and presentation by cells of the innate immune system (e.g., macrophages, dendritic cells) (Iwasaki & Medzhitov 2010). Thus, given the primary role of innate immunity in non-specifically fighting infections and in regulating acquired immune responses, it is likely that the innate immune processes are paramount in driving resistance to novel pathogens, particularly those that avoid immune detection.

Here we make use of the natural epizootic of conjunctivitis caused by the bacterium *Mycoplasma gallisepticum* (MG) in a North American songbird, the House finch (*Caropdacus mexicanus*) (Dhondt *et al.* 1998; Fischer *et al.* 1997), to investigate the

contribution of innate and acquired immunity to the evolution of resistance to a novel pathogen. Mycoplasmosis was first reported in house finches in Maryland in 1994 (Ley 1996). Following outbreak, the disease spread rapidly across eastern populations of house finches in North America. The severity of MG as a house finch pathogen early in the epizootic was confirmed by high mortality rates of naturally- and experimentally-infected finches maintained in captivity (Farmer *et al.* 2002; Luttrell *et al.* 1998; Roberts *et al.* 2001a). In the wild, hundreds of millions of birds were estimated to have died between 1994 and 1998 (Nolan *et al.* 1998), causing a significant decline in the abundance of house finches over the entire eastern portion of their range (Hochachka & Dhondt 2000). The prevalence of MG in house finches subsequently declined (Hartup *et al.* 2001; Roberts *et al.* 2001b) and evidence now suggests that MG has reached endemic levels in eastern North America, at least in part due to the spread of host resistance within 12 years of exposure to MG (Bonneaud *et al.* 2011).

Mycoplasma bacteria are known for effectively evading and manipulating host immune defenses (for a review see (Razin *et al.* 1998). For example, MG maintains a high diversity of cell surface molecules (Chambaud *et al.* 1999), including surface lipoproteins, and can vary its antigenic composition at the cell surface in response to environmental cues (Baseggio *et al.* 1996; Markham *et al.* 1998). Such antigenic variation allows mycoplasmas to be resistant to phagocytosis in susceptible hosts (Marshall *et al.* 1995). Immuno-modulatory effects include the ability to induce an inflammatory response at the site of infection (Ganapathy & Bradbury 2003; Gaunson *et al.* 2006), causing host lesions (Ley 2008), as well as the ability to suppress other components of host immunity (Javed *et al.* 2007). For example, simultaneous inoculation of poultry with MG and *Haemophilus gallinarum* (Matsuo *et al.*

1978) or avian pneumovirus (Naylor *et al.* 1992) has been found to lower the humoral antibody response to both *H. gallinarum* and pneumovirus in chickens and turkeys, respectively. Finally, MG infection is associated with suppressed T cell activity two weeks after infection (Ganapathy & Bradbury 2003; Gaunson *et al.* 2000).

To examine the contributions of innate and acquired immunity to the evolution of resistance to MG in house finches, we conducted an infection experiment and examined transcriptional responses elicited in the spleen, an important tissue for the organization of both innate and acquired immunity (Mebius & Kraal 2005). Infection with pathogens is known to induce transcriptional responses in hosts (Jenner & Young 2005) and such responses can differ between individuals displaying varying levels of resistance to infection (Marquis *et al.* 2008). Investigating differences in gene expression profiles between resistant and susceptible hosts in response to experimental infection might therefore offer new insights into the genetic basis underlying immunity (Sarson *et al.* 2008; van der Sar *et al.* 2009). In our study, finches originated from either eastern U.S. (Alabama) populations, which have coexisted with MG since the mid-1990s and show evidence of having evolved resistance, or western U.S. (Arizona) populations with no prior exposure to MG (Bonneaud *et al.* 2011). Gene expression changes between infected and control finches were measured three and 14 days after experimental infection. Although immune processes three and 14 days post-infection will generally reflect innate and acquired activity, respectively (Farmer *et al.* 2002; Gaunson *et al.* 2000; Hickman-Davis *et al.* 1998; Lai *et al.* 1987), the genes that underpin these processes may both be expressed sharply after, and continue throughout, infection (Caipang *et al.* 2009; Raida & Buchmann 2008; Sarson *et al.* 2008; van der Sar *et al.* 2009). Thus, investigating the role of innate and acquired immunity in the evolution of resistance to MG

using patterns of gene expression profiles in transcriptional responses to MG-infection will require testing predictions regarding temporal versus geographical differences.

We make two broad predictions regarding the role of innate and acquired immune responses in the evolution of resistance to MG in eastern house finches. First, our results would suggest that MG has selected on innate immunity if: (1) eastern and western populations differed in the transcriptional changes observed between control and MG-infected finches three days post-infection; (2) gene expression differences involved significant gene down-regulation in western but not eastern finches; and (3) genes associated with acquired immunity were up-regulated on day 14 only. These predictions arise because transcriptional differences between populations in the early stages of experimental infection would suggest that early-acting innate immune processes differ between populations, and down-regulation is expected in Arizona due to the immuno-modulatory effects of MG infection. However, this scenario would unambiguously support the hypothesis of selection on innate immunity only if genes known to be associated with acquired immunity were not differentially expressed at an early stage of infection. Second, by contrast, our results would suggest a sole role of acquired immunity in the resistance of eastern bird to MG if transcriptional changes only differed between populations fourteen days after infection and involved the up-regulation of genes associated with acquired immunity in eastern finches.

Material and Methods

Experimental infection

In January and February 2007, we captured male house finches from two geographically distant locations: southeastern Arizona in the western U.S. which was outside the 2007-range

of MG; and southern Alabama in the eastern U.S., where finches had co-existed with MG for 12 years. Sampling was conducted at 3 different suburban sites in both states: in Arizona, sites were 1-2 km apart and the birds were captured over 3 days; in Alabama, sites were 10-103 km apart and the birds were captured over 30 days. Following capture, birds were immediately transported by plane from Arizona ($N=37$) and by car within Alabama ($N=64$), and established in aviaries at Auburn University, Alabama. Finches were held in cages 0.5 m x 0.5 m with two birds per cage for the duration of the study. Cages were kept indoors, in temperature-controlled rooms with natural light through windows (day-length was unregulated but comparable to the locales from which the birds were captured). Captive finches were fed sunflower seed, brown and white millet, grit, and water *ad libitum*, as well as apple slices and crushed eggshells weekly. The housing conditions, food, and day-length regime were identical for birds from both populations, and represented novel conditions for birds from both populations.

To confirm that the finches had not been infected with MG prior to our study, individuals from Alabama and Arizona were quarantined in separate rooms for the first month. Following quarantine, birds were weighed (± 0.1 g) and had a blood sample taken via brachial venipuncture (~ 60 μ l of whole blood). Whole blood was tested for MG antibodies using serum plate agglutination assay (SPA), a reliable means of determining prior exposure to MG, (Luttrell *et al.* 1996). All birds in the study were further tested for exposure to MG via amplification of MG DNA from choanal and conjunctival swabs (Roberts *et al.* 2001a). Twelve birds from the Alabama population were removed from the experiment when they showed evidence of exposure to MG (8 were symptomatic at capture, 1 developed symptoms during quarantine, and 3 were seropositive for MG-antibodies). In addition, a further 9 from

Arizona and 20 from Alabama were used in a different experiment, leaving 28 Arizona birds and 32 Alabama birds in this study.

Birds were either kept as controls or infected via ocular inoculation with 20 μ l of culture containing 1×10^4 to 1×10^6 color changing units/ml of an early 2007 Auburn MG isolate. Control birds were sham infected using sterile SP4 medium (Whitcomb 1983). Control ($N=11$ birds from Arizona and 9 from Alabama) and infected birds were maintained under identical conditions, but in separate rooms of an aviary. Birds were euthanized three days ($N=6$ from Arizona and $N=11$ from Alabama) and 14 days ($N=11$ from Arizona and $N=12$ from Alabama) after treatment. The spleens and the conjunctiva from all birds were removed immediately after euthanization, stored in RNAlater (Ambion), and placed at -80°C .

Sample preparation and microarray hybridization and analysis

Molecular methods and analyses are detailed in Bonneaud et al (Bonneaud *et al.* 2011). Briefly, we extracted total RNA from approximately 17 mg of spleen tissue using Qiagen RNeasy miniprep spin columns and followed by DNase digestion of genomic DNA according to the manufacturers' protocols. We determined the quantity of purified total RNA using a Nanodrop spectrophotometer and determined RNA integrity on an Agilent 2100 Bioanalyzer. All RNA extracts were stored at -80°C until further processing.

The samples were hybridized onto a microarray printed with a selection of cDNA clones from two subtraction suppression hybridization libraries (Bonneaud *et al.* 2011). These libraries are enriched in clones differentially expressed between MG-infected and control house finches 2-weeks post-infection ($N=16,512$ clones) (Wang et al. 2006). Using libraries

enriched in cDNA differentially expressed 14 days post-infection increases the probability that both innate and acquired immune processes have been activated (Janeway 2005). Of all the clones present in the libraries, 220 were previously identified as significantly differentially expressed between infected and controls using a macroarray approach (Wang *et al.* 2006). The microarray consisted of unique amplicons of these 220 clones, as well as amplicons of 694 randomly selected clones from the enriched libraries (Bonneaud *et al.* 2011). Additionally, it contained five house finch housekeeping genes (*Actin related protein 2/3*, *ATP synthetase*, *ATPase V1 subunit G1*, *Basic transcription factor 3*, *Calmodulin 2*) and 11 *E. coli* housekeeping genes (*arcA*, *aroE*, *dnaE*, *gapA*, *gnd*, *icdA*, *pgm*, *polB*, *putin*, *trpA*, *trpB*; (Hommais *et al.* 2005; Noller *et al.* 2003)) to facilitate normalization procedures. All clones were printed twice on each grid and each grid was replicated twice on each half microarray slide. We used a common reference design (Yang & Speed 2002), in which we pooled 2 to 5 spleens from birds from the same population in the same treatment to generate enough mRNA for microarray hybridizations and hybridized two pools for each treatment from each population. Pools were labeled using Cy5 dye and hybridized against a common reference, made by pooling an aliquot of all the individual samples from all treatments and labeled with Cy3.

We used the software package GenePix to yield log base-2 (\log_2) measurements for mean fluorescence intensities for each dye channel in each spot on the array and to flag low quality spots. We normalized the log base-2 measurements of mean fluorescence intensities for each dye channel in each spot on the array using R software (<http://www.r-project.org>), and a Matlab interface (MArray), which allows results to be graphically presented and normalized (Wang *et al.* 2002). Normalized signal ratios were then fitted to Linear Model for Microarray

data (LIMMA) in an R Bioconductor package; LIMMA is similar to a General Linear Model but provides False Discovery Rate (FDR)-adjusted probability values of differential expression. This approach controls for multiple comparisons in microarray data, substantially reducing the probability of discovering false positives (Type I errors) (Benjamini & Hochberg 1995). The ratios generated by the external spike-ins were used for quality control. To control for within-hybridization spatial variation, we compared the signal from the 2 replicated grids. To control for between-slide differences, we compared the signals from the *E. coli* external spike-ins, the house finch housekeeping genes and the common reference on the different slides. All clones were considered to be differentially expressed only when both replicates on the array displayed a significant deviation from the mean of the standard. All differentially expressed clones were sequenced on an ABI 377 sequencer. Forward and reverse sequences generating a BLAST hit with an e-value $< 1 \times 10^{-20}$ and with more than 100 nucleotides were categorized by their vertebrate homologues, while all other genes were considered to be unknown. Gene ontology category and function were determined using Harvester (<http://harvester.fzk.de/harvester/>).

Comparisons

To test our predictions, we made four comparisons of transcriptional responses to MG-infection between finches (Figure 1A). We compared expression differences between infected birds on day three post-infection vs. controls in Arizona (1) and Alabama (2), as well as those differences between control and experimental birds on day 3 with those on day 14 in Arizona (3) and Alabama (4). Differences in gene expression patterns were analyzed using comparisons of observed versus expected frequencies in binomial test and contingency tables (comparing two or more than two independent frequencies, respectively) or McNemar's and

Cochran's Q test (when comparing two, or more than two, non-independent frequencies, respectively; e.g. when frequencies are based on the same sample of subjects or matched-pair samples such as before and after treatment).

Results

We found 105 clones that were significantly differentially expressed in this study, of which 73 were differentially expressed three days after infection and 99 were differentially expressed 14 days after infection. Sequencing these clones revealed 25 vertebrate orthologs (Figure 1B): 13 and 24 which were differentially expressed three and 14 days after infection, respectively. All other clones were unknown. Gene ontology categories and primary functions of the 25 genes included immunity (6 genes), redox metabolism (3), metabolism (1), signal transduction (4), stress (1), cytoskeleton (4), transcription/translation (3), transport (2), and cell differentiation (1). Given that all of these genes are differentially expressed as a result of experimental infection, it is likely they all play some role in the response to infection. Indeed, in addition to the 6 genes with direct immune function (*T-cell immunoglobulin and mucin domain containing 4*, *MHC class II-associated invariant chain*, *programmed death ligand 1*, *lectin galactoside-binding soluble 2 protein*, *neutrophil cytosolic factor 4*, *complement factor H*), three of the 'non-immune' genes above have been shown to have auxiliary immune function (*thioredoxin* (Nordberg & Arner 2001), *RhoA GTPase* (Scheele *et al.* 2007), *lymphocyte cytosolic protein* (Samstag *et al.* 2003)) (Figure 1B). We can rule out the possibility that our results are due to differences in cDNA quality or abundance between samples due to our extensive use of within-and between-slide controls (see Methods).

All predictions that selection has acted on innate immunity only, or on both innate and acquired immunity, were upheld. Three days post-infection, 13 of the 25 genes identified displayed significant differences in expression between infected and control birds from Arizona (comparison 1), but none did between such birds from Alabama (comparison 2) (Figure 1B, C) (two-sample binomial test=3.85, $P<0.001$). In addition, 85% of those 13 genes differentially expressed on day three between infected and control birds in Arizona were down-regulated (one-sample binomial test, $P = 0.02$; Figure 1B, C). Finally, in Arizona birds, gene expression profiles between experimental and control birds remained similar on days three and 14 post-treatment (13 genes differentially expressed in comparison 1 and 20 in comparison 3: two-sample binomial test=-1.48, $P=0.14$), and there was no change in the proportion of genes that were down-regulated between the two time points (McNemar's test, $\chi^2=0.14$, $P = 0.71$). By contrast, in Alabama, a significantly greater number of genes were expressed in infected birds on day 14 than on day three (0 genes in comparison 2, 11 in comparison 4: two-sample binomial test=-3.51, $P<0.001$), and eight of these 14 genes were up-regulated. Importantly, of these eight genes differentially expressed on day 14 in Alabama, one was identified as having a role in innate immunity (*neutrophil cytosolic factor 4*) and two in acquired immunity (*T-cell immunoglobulin* and *MHC class II associated invariant chain*), and none was differentially expressed on day 3. This latter result means that population differences in expression patterns 3 days post-infection are unlikely to be attributed to acquired immune processes.

Discussion

We have shown recently that eastern U.S. populations of house finches evolved resistance to a devastating outbreak of MG over a 12-year period (Bonneaud *et al.* 2011). Here we use

microarray analysis and experimental infections in finches from MG-exposed eastern U.S. (Alabama) and unexposed western U.S. (Arizona) populations to investigate whether changes to innate and/or acquired immunity have accompanied this evolutionary event. Relative to controls, gene expression profiles of birds from Arizona versus Alabama differed both three and 14 days following experimental infection, with infected birds from Arizona showing significant down-regulation of gene expression patterns on both days compared to those from Alabama. Moreover, while gene expression profiles were similar on days three and 14 in Arizona finches, in Alabama finches, profiles differed significantly between day three and 14. This change in gene expression patterns in Alabama finches was generated by the up-regulation of acquired immune processes by day 14 but not on day three. Inter-population differences between infected and control birds on days three and 14 were therefore likely due to differences in innate and acquired immune activity. From these observations, we suggest that mutations affecting innate immunity only, or both innate and acquired immunity, have accompanied the evolution of resistance to MG.

The conclusion that mutations affecting innate immunity played a role in the evolution of resistance to MG is based on our upholding of three predictions (see Introduction). These were: (1) relative to controls, infected birds from Arizona and Alabama displayed distinct transcriptional responses in the early stages of experimental infection; (2) expression patterns in Alabama were consistent with increased resistance to MG; and (3) genes associated with acquired immunity were only up-regulated after population differences in transcription were first observed. These observations also allowed us to reject the hypothesis that mutations associated with acquired immunity alone led to the evolution of resistance to MG among eastern U.S. house finches. Nevertheless, mutations associated with acquired immune

processes, in addition to those associated with innate immune processes, may have played a role in the evolution of resistance, as evidenced by the transcriptional differences of infected versus control birds between the two populations on day 14, and within Alabama between days three and 14.

Evidence from laboratory mice and rats also suggests a role of both innate and acquired immunity in fighting infections with *Mycoplasmas*, but with innate immunity playing a predominant role in fighting initial infections (Hickman-Davis 2002). For example, while acquired immunity appears to be implicated in controlling the spread of *M. pulmonis* within the body, innate immunity is important for resistance against acute infections (Cartner *et al.* 1998). Natural killer cells and macrophages, which are important actors of innate immunity, have been shown to play important roles in conferring resistance to *M. pulmonis* (Hickman-Davis *et al.* 1997; Lai *et al.* 1990). In addition, phagocytosis, bacterial killing and the release of reactive nitrogen species by macrophages during *M. pulmonis* and *M. pneumonia* infections seem to be facilitated by collectins, such as surfactant-associated proteins A (Hickman-Davis *et al.* 1998; Kalina *et al.* 2000; Marshall *et al.* 1995), which represent a major group of pattern recognition proteins of the innate immune system (van de Wetering *et al.* 2004). Surfactant-associated proteins A are encoded by polymorphic genes (reviewed in (Floros *et al.* 2009; Ledford *et al.*), and both limit inflammatory responses and interact with T cells, making them particularly interesting candidate genes to examine in the context of the evolution of resistance to MG in eastern U.S. house finches.

Although studies of the response of mammalian hosts to *Mycoplasmas* suggest a role of both innate and acquired immunity in conferring resistance, the evolutionary origins of resistance

355 to MG could be associated with changes in gene(s) implicated in innate immunity only, given
356 that innate immune processes both precede and play a critical role in the activation of
357 acquired processes (Iwasaki & Medzhitov 2010). Under this hypothesis, any population
358 differences in acquired immunity may simply be a consequence of differences in innate
359 immune activity. Hence, although we are not in a position to distinguish whether mutations
360 associated with innate, or with both innate and acquired, immunity have led to the evolution
361 of resistance to MG in eastern house finches, the transcriptional differences we observed on
362 day 14 may result from a single mutation affecting innate immunity and allowing eastern
363 finches to subsequently trigger an acquired immune response. Our results are reminiscent of
364 those obtained from similar experimental infections of wild rabbits with the *myxoma* virus
365 (Best & Kerr 2000). Resistant rabbits had elevated immune responses within four days post-
366 infection, in advance of the subsequent increased cell-mediated immune response at least six
367 days after infection. The increased resistance of populations of rabbits having experienced
368 the *myxomatosis* outbreak was therefore hypothesized to be mediated by enhanced innate
369 immune activity, which subsequently allowed the development of a specific cell-mediated
370 immune response (Best & Kerr 2000). While mutations arising in genes associated with both
371 innate and acquired immunity may have been subject to natural selection, a more
372 parsimonious scenario may be that a change in the frequency of a single mutation affecting
373 innate immune processes has been primarily responsible for the evolution of resistance to
374 MG. The speed with which resistance evolved in eastern house finches (Bonneaud *et al.*
375 2011) and the rarity of mutations conferring phenotypic advantages in evolving populations
376 (Blount *et al.* 2008), suggests that selection is unlikely to have simultaneously favored the
377 spread of two or more distinct pre-existing alleles, but further work is required to test this
378 hypothesis.

379

380 Although all of the transcriptional changes that we observed occurred in response to the
381 experimental infection, and hence might play a role in resistance, we identified six genes that
382 are known to have a direct role in immunity in model organisms and humans, and three genes
383 known to play an auxiliary role in immunity (see Table 1 for full details of gene functions
384 and associated references). Of the six immune genes, three encode proteins that are directly
385 involved in innate immunity and implicated in phagocytosis-induced superoxide production
386 and/or control of inflammation or complement-mediated immunity: *neutrophil cytosolic*
387 *factor 4* was up-regulated on day 14 in Alabama finches; *lectin galactoside-binding soluble 2*
388 *protein (galectin)* was down-regulated on day 14 in Arizona finches; and *complement factor*
389 *H* was up-regulated on day 14 in Arizona finches; surprisingly, none was differentially
390 expressed on day three. In addition to the *galectin* gene above which also has direct
391 involvement in acquired immunity, *T-cell immunoglobulin and mucin domain containing 4*,
392 which plays a role in T-cell activation, was up-regulated in Alabama finches on day 14,
393 *programmed death ligand 1*, which regulates T-cell activation and tolerance, was down-
394 regulated in Arizona finches on day 14, and *MHC class II-associated invariant chain Ii*,
395 which plays a role in the assembly of MHC class II molecules, was up-regulated in Alabama
396 finches on day 14 and down-regulated in Arizona on both days. Finally, *thioredoxin* and
397 *RhoA GTPase* which both have auxiliary function in innate immunity (antioxidant activities,
398 regulation and coordination of the innate immune response, respectively) were down
399 regulated on days three and 14 in Arizona finches and up-regulated in Alabama finches on
400 day 14, while *lymphocyte cytosolic protein*, which has auxiliary function in acquired
401 immunity (stabilization of actin filaments during T-cell migration) was down-regulated in
402 Arizona finches on both days and up-regulated in Alabama finches on day 14.

MG infection is known to cause the suppression of certain immune components in the initial stages of infection in chickens as evidenced by the significant down-regulation of cytokines (CCL20, IL8 and IL12) as early as 24 hours after exposure (Mohammed *et al.* 2007). These effects can last up to 8 days following infection (Mohammed *et al.* 2007). The expression profiles above suggest that Arizona finches were immune-suppressed throughout the experimental infection, with the majority of genes being down-regulated, including 3 genes associated with immunity on day three and five on day 14. Interestingly, the only immune gene that was up-regulated in infected finches from Arizona (*complement factor H*) has been found to restrict the activation of the complement cascade in humans (de Cordoba & de Jorge 2008) and hence exhibits a direction of expression change consistent with the suppression of immune activity. Conversely, Alabama finches displayed evidence of resistance to immune manipulation as no immune related genes were down-regulated on day three or day 14. Finally, in line with the study of the rabbits/myxomatosis system wherein immunity against myxomatosis was associated with increased cell-mediated (i.e. T helper-cell activity) rather than humoral (i.e. antibody) responses (Best and Kerr 2000), we found that the two immune genes up-regulated on day 14 in Alabama were associated with cell-mediated immunity and that no differentially expressed genes identified were associated with humoral responses. Thus, our results suggest that resistance to MG evolved in the eastern U.S. via the ability to mount an innate immune response followed by a cell-mediated immune response against MG.

Resistance can evolve via increased host ability to physiologically limit pathogen invasion upon contact (avoidance), to clear infections (recovery), or to suffer the costs associated with

the presence of the pathogen (tolerance) (Boots & Bowers 1999). Whether clearance of infection is mediated by innate or acquired immune processes should depend on characteristics of both the host and the pathogen, such as host lifespan, pathogen transmission rate and pathogenecity, and host recovery rate (Boots & Bowers 2004). In the initial stages of a novel and severe epizootic outbreak, however, innate immune mechanisms conferring increased resistance may be the target of selection, even if natural selection ultimately leads to the evolution of highly-specific acquired immune processes. Our results highlight the importance of identifying not only the genetic correlates of adaptation, but also the molecular and cellular processes underlying phenotypic change to better understand how wild populations respond to natural selection (Manceau *et al.* 2011; Shapiro *et al.* 2004). In addition, we showed that the same immune processes appear to be adopted by different species in response to related pathogens, suggesting that the pathways favored by natural selection may be analogous across taxa. Finally, although previous studies of temporal transcriptional changes have been used to identify the immune processes associated with increased resistance to infectious diseases in both domestic and laboratory animals (Raida and Buchmann 2008; Sarson *et al.* 2008), ours is the first to do so in a wild population known to have evolved disease resistance under pathogen-driven natural selection.

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Literature cited

- Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife immunogenetics? *Trends in Ecology & Evolution*, **21**, 433-438.
- Baseggio N, Glew MD, Markham PF, Whithear KG, Browning GF (1996) Size and genomic location of the pMGA multigene family of *Mycoplasma gallisepticum*. *Microbiology-Uk*, **142**, 1429-1435.
- Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B-Methodological*, **57**, 289-300.
- Benning TL, LaPointe D, Atkinson CT, Vitousek PM (2002) Interactions of climate change with biological invasions and land use in the Hawaiian Islands: Modeling the fate of endemic birds using a geographic information system. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 14246-14249.
- Best SM, Kerr PJ (2000) Coevolution of host and virus: The pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. *Virology*, **267**, 36-48.

474 Blount ZD, Borland CZ, Lenski RE (2008) Historical contingency and the evolution of a key
 475 innovation in an experimental population of *Escherichia coli*. *Proceedings of the*
 476 *National Academy of Sciences of the United States of America*, **105**, 7899-7906.

477 Bonneaud C, Balenger S, Russell AF, *et al.* (2011) Rapid evolution of disease resistance is
 478 accompanied by functional changes in gene expression in a wild bird. *Proceedings of*
 479 *the National Academy of Sciences of the United States of America*, **108**, 7866-7871.

480 Boots M, Bowers RG (1999) Three mechanisms of host resistance to microparasites -
 481 Avoidance, recovery and tolerance - Show different evolutionary dynamics. *Journal*
 482 *of Theoretical Biology*, **201**, 13-23.

483 Boots M, Bowers RG (2004) The evolution of resistance through costly acquired immunity.
 484 *Proceedings of the Royal Society of London Series B-Biological Sciences*, **271**, 715-
 485 723.

486 Caipang CMA, Brinchmann MF, Kiron V (2009) Profiling gene expression in the spleen of
 487 Atlantic cod, *Gadus morhua* upon vaccination with *Vibrio anguillarum* antigen.
 488 *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*,
 489 **153**, 261-267.

490 Cartner SC, Lindsey JR, Gibbs-Erwin J, Cassell GH, Simecka JW (1998) Roles of innate and
 491 adaptive immunity in respiratory mycoplasmosis. *Infection and Immunity*, **66**, 3485-
 492 3491.

493 Chambaud I, Wroblewski H, Blanchard A (1999) Interactions between mycoplasma
 494 lipoproteins and the host immune system. *Trends in Microbiology*, **7**, 493-499.

495 de Cordoba SR, de Jorge EG (2008) Translational mini-review series on complement factor
 496 H: Genetics and disease associations of human complement factor H. *Clinical and*
 497 *Experimental Immunology*, **151**, 1-13.

498 Dhondt AA, Tessaglia DL, Slothower RL (1998) Epidemic mycoplasmal conjunctivitis in
 499 house finches from Eastern North America. *Journal of Wildlife Diseases*, **34**, 265-
 500 280.

501 Diamond J (1997) *Guns, germs, and steel* W. W. Norton & Company, New York, U.S.A.

502 Farmer KL, Hill GE, Roberts SR (2002) Susceptibility of a naïve population of house finches
 503 to *Mycoplasma gallisepticum*. *Journal of Wildlife Diseases*, **38**, 282-286.

504 Fischer JR, Stallknecht DE, Luttrell MP, Dhondt AA, Converse KA (1997) Mycoplasmal
 505 conjunctivitis in wild songbirds: The spread of a new contagious disease in a mobile
 506 host population. *Emerging Infectious Diseases*, **3**, 69-72.

507 Floros J, Wang GR, Mikerov AN (2009) Genetic complexity of the human innate host
 508 defense molecules, surfactant protein A1 (SP-A1) and SP-A2-impact on function.
 509 *Critical Reviews in Eukaryotic Gene Expression*, **19**, 125-137.

510 Ganapathy K, Bradbury JM (2003) Effects of cyclosporin A on the immune responses and
 511 pathogenesis of a virulent strain of *Mycoplasma gallisepticum* in chickens. *Avian*
 512 *Pathology*, **32**, 495-502.

513 Gaunson JE, Philip CJ, Whithear KG, Browning GF (2000) Lymphocytic infiltration in the
 514 chicken trachea in response to *Mycoplasma gallisepticum* infection. *Microbiology-Uk*,
 515 **146**, 1223-1229.

516 Gaunson JE, Philip CJ, Whithear KG, Browning GF (2006) The cellular immune response in
 517 the tracheal mucosa to *Mycoplasma gallisepticum* in vaccinated and unvaccinated
 518 chickens in the acute and chronic stages of disease. *Vaccine*, **24**, 2627-2633.

519 Grenfell BT, Dobson AP (1995) *Ecology of infectious diseases in natural populations*
 520 Cambridge University Press, Cambridge.

521 Haldane JBS (1949) Disease and evolution. *La Ricerca Scientifica Supplemento A*, **19**, 68-76.

522 Hartup BK, Bickal JM, Dhondt AA, Ley DH, Kollias GV (2001) Dynamics of conjunctivitis
523 and *Mycoplasma gallisepticum* infections in house finches. *Auk*, **118**, 327-333.

524 Hedrick PW (2002) Pathogen resistance and genetic variation at MHC loci. *Evolution*, **56**,
525 1902-1908.

526 Hickman-Davis JM (2002) Role of innate immunity in respiratory mycoplasma infection.
527 *Frontiers in Bioscience*, **7**, D1347-D1355.

528 Hickman-Davis JM, Lindsey JR, Zhu S, Matalon S (1998) Surfactant protein A mediates
529 mycoplasmacidal activity of alveolar macrophages. *American Journal of Physiology-
530 Lung Cellular and Molecular Physiology*, **274**, L270-L277.

531 Hickman-Davis JM, Michalek SM, Gibbs-Erwin J, Lindsey JR (1997) Depletion of alveolar
532 macrophages exacerbates respiratory mycoplasmosis in mycoplasma-resistant C57BL
533 mice but not mycoplasma-susceptible C3H mice. *Infection and Immunity*, **65**, 2278-
534 2282.

535 Hochachka WM, Dhondt AA (2000) Density-dependent decline of host abundance resulting
536 from a new infectious disease. *Proceedings of the National Academy of Sciences of
537 the United States of America*, **97**, 5303-5306.

538 Hommais F, Pereira S, Acquaviva C, Escobar-Paramo P, Denamur E (2005) Single-
539 nucleotide polymorphism phylotyping of *Escherichia coli*. *Applied and
540 Environmental Microbiology*, **71**, 4784-4792.

541 Iwasaki A, Medzhitov R (2010) Regulation of adaptive immunity by the innate immune
542 system. *Science*, **327**, 291-295.

543 Janeway C (2005) *Immunobiology : the immune system in health and disease* Garland
544 Science, New York :.

545 Janeway CA (1989) Approaching the asymptote - evolution and revolution in immunology.
 546 *Cold Spring Harbor Symposia on Quantitative Biology*, **54**, 1-13.

547 Javed M, Frasca S, Cecchini K, *et al.* (2007) Chemokine and cytokine gene expression
 548 profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5.
 549 *Vaccine*, **25**, 8611-8621.

550 Jenner RG, Young RA (2005) Insights into host responses against pathogens from
 551 transcriptional profiling. *Nature Reviews Microbiology*, **3**, 281-294.

552 Kalina M, Blau H, Riklis S, Hoffman V (2000) Modulation of nitric oxide production by lung
 553 surfactant in alveolar macrophages. In: *Biology and Pathology of Innate Immunity*
 554 *Mechanisms* (eds. Keisari Y, Ofek I), pp. 37-48.

555 Kerr PJ, Best SM (1998) Myxoma virus in rabbits. *Revue Scientifique Et Technique De L*
 556 *Office International Des Epizooties*, **17**, 256-268.

557 Lai WC, Bennett M, Pakes SP, *et al.* (1990) Resistance to *Mycoplasma pulmonis* mediated by
 558 activated natural-killer-cells. *Journal of Infectious Diseases*, **161**, 1269-1275.

559 Lai WC, Pakes SP, Lu YS, Brayton CF (1987) *Mycoplasma pulmonis* infection augments
 560 natural-killer cell-activity in mice. *Laboratory Animal Science*, **37**, 299-303.

561 Ledford JG, Pastva AM, Wright JR (2010) Collectins link innate and adaptive immunity in
 562 allergic airway disease. *Innate Immunity*, **16**, 183-190.

563 Ley DH (2008) *Mycoplasma gallisepticum* Infection. In: *Diseases of Poultry* (ed. Saif YM),
 564 pp. 722-743. Iowa State Press, Ames, U.S.A.

565 Ley DH, Berkhoff, J.E., McLaren, J.M. (1996) Isolation of *Mycoplasma gallisepticum* in
 566 house finches with conjunctivitis. *Avian Diseases*, **40**, 480-483.

567 Lips KR, Brem F, Brenes R, *et al.* (2006) Emerging infectious disease and the loss of
 568 biodiversity in a Neotropical amphibian community. *Proceedings of the National*
 569 *Academy of Sciences of the United States of America*, **103**, 3165-3170.

570 Luttrell MP, Fischer JR, Stallknecht DE, Kleven SH (1996) Field investigation of
 571 *Mycoplasma gallisepticum* infections in house finches (*Carpodacus mexicanus*) from
 572 Maryland and Georgia. *Avian Diseases.*, **40**, 335-341.

573 Luttrell MP, Stallknecht DE, Fischer JR, Sewell CT, Kleven SH (1998) Natural *Mycoplasma*
 574 *gallisepticum* infection in a captive flock of house finches. *Journal of Wildlife*
 575 *Diseases*, **34**, 289-296.

576 Manceau M, Domingues VS, Mallarino R, Hoekstra HE (2011) The Developmental Role of
 577 Agouti in Color Pattern Evolution. *Science*, **331**, 1062-1065.

578 Markham PF, Glew MD, Browning GF, Whithear KG, Walker ID (1998) Expression of two
 579 members of the pMGA gene family of *Mycoplasma gallisepticum* oscillates and is
 580 influenced by pMGA-specific antibodies. *Infection and Immunity*, **66**, 2845-2853.

581 Marquis JF, Nantel A, LaCourse R, *et al.* (2008) Fibrotic response as a distinguishing feature
 582 of resistance and susceptibility to pulmonary infection with *Mycobacterium*
 583 *tuberculosis* in mice. *Infection and Immunity*, **76**, 78-88.

584 Marshall AJ, Miles RJ, Richards L (1995) The Phagocytosis of Mycoplasmas. *Journal of*
 585 *Medical Microbiology*, **43**, 239-250.

586 Marshall ID, Fenner F (1958) Studies in the epidemiology of infectious myxomatosis of
 587 rabbits. V. Changes in the innate resistance of wild rabbits between 1951 and 1959.
 588 *Journal of Hygiene*, **56**, 288-302.

589 Matsuo K, Kuniyasu C, Yamada S, Susumi S, Yamamoto S (1978) Suppression of immuno-
590 responses to *Hemophilus gallinarum* with nonviable *Mycoplasma gallisepticum* in
591 chickens. *Avian Diseases*, **22**, 552-561.

592 Mebius RE, Kraal G (2005) Structure and function of the spleen. *Nature Reviews*
593 *Immunology*, **5**, 606-616.

594 Mohammed J, Frasca S, Cecchini K, *et al.* (2007) Chemokine and cytokine gene expression
595 profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5.
596 *Vaccine*, **25**, 8611-8621.

597 Naylor CJ, Alankari AR, Alafaleq AI, Bradbury JM, Jones RC (1992) Exacerbation of
598 *Mycoplasma gallisepticum* Infection in Turkeys by Rhinotracheitis Virus. *Avian*
599 *Pathology*, **21**, 295-305.

600 Nolan PM, Hill GE, Stoeckl AM (1998) Sex, size, and plumage redness predict house finch
601 survival in an epidemic. *Proceedings of the Royal Society of London Series B-*
602 *Biological Sciences*, **265**, 961-965.

603 Noller AC, McEllistrem MC, Stine OC, *et al.* (2003) Multilocus sequence typing reveals a
604 lack of diversity among *Escherichia coli* O157 : H7 isolates that are distinct by
605 pulsed-field gel electrophoresis. *Journal of Clinical Microbiology*, **41**, 675-679.

606 Nordberg J, Arner ESJ (2001) Reactive oxygen species, antioxidants, and the mammalian
607 thioredoxin system. *Free Radical Biology and Medicine*, **31**, 1287-1312.

608 Pierny SB, Oliver MK (2006) The evolutionary ecology of the major histocompatibility
609 complex. *Heredity*, **96**, 7-21.

610 Raida MK, Buchmann K (2008) Development of adaptive immunity in rainbow trout,
611 *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri*. *Fish*
612 *& Shellfish Immunology*, **25**, 533-541.

613 Razin S, Yogev D, Naot Y (1998) Molecular biology and pathogenicity of mycoplasmas.
614 *Microbiology and Molecular Biology Reviews*, **62**, 1094-1156.

615 Roberts SR, Nolan PM, Hill GE (2001a) Characterization of *Mycoplasma gallisepticum*
616 infection in captive house finches (*Carpodacus mexicanus*) in 1998. *Avian Diseases*,
617 **45**, 70-75.

618 Roberts SR, Nolan PM, Lauerman LH, Li LQ, Hill GE (2001b) Characterization of the
619 mycoplasmal conjunctivitis epizootic in a house finch population in the southeastern
620 USA. *Journal of Wildlife Diseases*, **37**, 82-88.

621 Samstag Y, Eibert SM, Klemke M, Wabnitz GH (2003) Actin cytoskeletal dynamics in T
622 lymphocyte activation and migration. *Journal of Leukocyte Biology*, **73**, 30-48.

623 Sarson AJ, Parvizi P, Lepp D, Quinton M, Sharif S (2008) Transcriptional analysis of host
624 responses to Marek's disease virus infection in genetically resistant and susceptible
625 chickens. *Animal Genetics*, **39**, 232-240.

626 Scheele JS, Marks RE, Boss GR (2007) Signaling by small GTPases in the immune system.
627 *Immunological Reviews*, **218**, 92-101.

628 Shapiro MD, Marks ME, Peichel CL, *et al.* (2004) Genetic and developmental basis of
629 evolutionary pelvic reduction in threespine sticklebacks. *Nature*, **428**, 717-723.

630 Sommer S (2005) The importance of immune gene variability (MHC) in evolutionary
631 ecology and conservation. *Frontiers in Zoology*, **2**, 16.

632 Spurgin LG, Richardson DS (2010) How pathogens drive genetic diversity: MHC,
633 mechanisms and misunderstandings. *Proceedings of the Royal Society B-Biological*
634 *Sciences*, **277**, 979-988.

635 van de Wetering JK, van Golde LMG, Batenburg JJ (2004) Collectins - Players of the innate
636 immune system. *European Journal of Biochemistry*, **271**, 1229-1249.

637 van der Most PJ, de Jong B, Parmentier HK, Verhulst S (2011) Trade-off between growth
 638 and immune function: a meta-analysis of selection experiments. *Functional Ecology*,
 639 **25**, 74-80.

640 van der Sar AM, Spaink HP, Zakrzewska A, Bitter W, Meijer AH (2009) Specificity of the
 641 zebrafish host transcriptome response to acute and chronic mycobacterial infection
 642 and the role of innate and adaptive immune components. *Molecular Immunology*, **46**,
 643 2317-2332.

644 Wang JB, Nygaard V, Smith-Sorensen B, Hovig E, Myklebost O (2002) MArray: analysing
 645 single, replicated or reversed microarray experiments. *Bioinformatics*, **18**, 1139-1140.

646 Wang Z, Farmer K, Hill G, Edwards SV (2006) A cDNA macroarray approach to parasite-
 647 induced gene expression in a songbird host: genetic response of House Finches to
 648 experimental infection by *Mycoplasma gallisepticum*. *Molecular Ecology*, **15**, 1263-
 649 1273.

650 Whitcomb RF (1983) Culture media for spiroplasmas. In: *Methods in mycoplasmaology* (eds.
 651 Razin S, Tully JG), pp. 147-158. Academic Press, New York, New York.

652 Wright S (1955) Classification of the Factors of Evolution. *Cold Spring Harbor Symposia on*
 653 *Quantitative Biology*, **20**, 16-D24.

654 Yang YH, Speed T (2002) Design issues for cDNA microarray experiments. *Nature Reviews*
 655 *Genetics*, **3**, 579-588.

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Figure legends

Figure 1: Comparisons and patterns of splenic gene expression. (A) Schematic of the analytical comparisons made: (1) infected on day three post-inoculation vs. controls in MG-unexposed Arizona; (2) infected on three post-inoculation vs. controls in MG-exposed Alabama; (3) infected on day fourteen post-inoculation vs. controls in Arizona; (4) infected day fourteen post-inoculation vs. controls in Alabama. Comparisons (3) and (4) were previously published in Bonneaud et al (2011). (B) Heat map of gene expression patterns for the 25 genes in comparisons 1-4 above (1st treatment/population vs. 2nd one). The 25 genes are all those showing differential expression in at least one comparison (1-4) and of known function. Values in red and green indicate significantly higher and lower expression levels, respectively, in comparisons 1-4 above, with bright colors reflecting at least a 3-fold difference in magnitude and values in black indicating no difference. Gene functions and identities are shown on the right; asterisks indicate genes with an identified auxiliary immune function. (C) Total number of genes of known function up-regulated (black) and down-regulated (white) in infected vs. control finches in the comparisons 1-4 above.

673 **Fig. 1**

